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High Human Papillomavirus DNA loads in Inflammatory Middle Ear Diseases

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Abstract:

Background. Previous studies reported human papillomaviruses (HPVs) in middle ear tumors, whereas these viruses have been poorly investigated in chronic inflammatory middle ear diseases. The purpose of this study was to investigate HPVs in non-tumor middle ear diseases, including chronic otitis media (COM). **Methods**. COM specimens (n=52), including chronic suppurative otitis media (CSOM) (n=38) and cholesteatoma (COMC) (n=14), as well as normal middle ear specimens (NME) (n=56) were analyzed. HPV DNA sequences and DNA loads were analyzed by quantitative PCR. HPV genotyping was performed by direct sequencing of the amplimers. **Results**. HPV DNA was detected in 23% (12/52) of COM and in 30.4% (17/56) NME ($p>0.05$). Specifically, HPV DNA sequences were revealed in 26.3% (10/38) of CSOM and in 14.3% (2/14) COMC ($p>.05$). Interestingly, the HPV DNA load was higher in COMC (mean 7.47 copy/cell) than in CSOM (mean 1.02 copy/cell), and NME (mean 1.18 copy/cell) ($P=.03$ and $P=.017$ versus CSOM and NME, respectively). HPV16 and HPV18 were the main genotypes detected in COMC, CSOM and NME. **Conclusions**. This data indicates that HPV-positive CSOM and COMC are generally associated with higher viral DNA loads as compared to NME. In addition, for the first time, HPVs were detected in normal middle ear mucosa specimens. This result suggests that NME is an additional epithelial tissue that can be HPV infected.

Keywords: HPV; infection; viral DNA load; inflammation; middle ear; chronic otitis media

1. Introduction

HPV infection is often associated to benign diseases and malignant tumors affecting the upper respiratory tract, including respiratory papillomatosis and oropharyngeal cancers [1,2]**.** Several studies also reported the HPV involvement in the development of middle ear squamous cell carcinoma [3–9]. Few studies are currently available for HPV in non-tumor middle ear diseases, such as chronic otitis media (COM), including chronic suppurative otitis media (CSOM) and chronic otitis media with cholesteatoma (COMC) [3–10].

CSOM is a middle ear disease relies on chronic inflammation. Different pro-inflammatory cytokines, such as TNF-α, IL-1β, IFN-γ, IL-6, have been found to be up-regulated in the middle ear mucosa sampled from CSOM patients [11]. However, the etiology of CSOM remains to be determined. The relationship between HPV infection and inflammation has been previously

reported [12]. It has been shown that persistent infection of high risk HPVs leads to the increase of proinflammatory cytokines, including IL-6, TNF-α, and MIP-1α [13]. In addition, high-risk HPV type 16 (HPV16) is able to increase the expression of cyclooxygenase- (COX-) 2, a key enzyme in the synthesis of prostaglandins, which are important mediators of inflammation [14,15]. Until now, only a single study reported HPV DNA sequences in CSOM, whereby different HPV genotypes, as HPV16, HPV18 and HPV6, have been detected in 30.7% of CSOM [4].

COMC is a form of expanding growth consisting of keratinizing squamous cell epithelium [16]. There is a great interest into the etiopathogenesis of HPV-associated cholesteatoma because HPV commonly infects the stratified epithelium [17,18]. However, conflicting data have been reported for HPV in COMC [10,19–21]. HPV sequences have been detected in COMC at different prevalence, ranging from 3% to 70% [10,19–21]. Moreover, no specific HPV genotypes have been associated to COMC, as high- and low-risk HPVs, such as HPV16, HPV18 and HPV6 and HPV11, have been detected [10,19–21].

One emerging evidence is that HPV infection can occur in different anatomical sites. Since HPV infects epithelia [22], all anatomical sites covered with epithelial tissues are potentially exposed to HPV infection. Apart from pluristratified tissues of cervix [23], vulva [24], and oral-pharynx [25], HPV sequences have been detected in simple epithelia from several anatomical districts, such as lung [26], upper respiratory tract [27], larynx [28] and nose [29]. Since the middle ear mucosa is composed of respiratory epithelium, and it is connected with the Eustachian tube to the oral and respiratory regions, HPV infection may also occur in the middle ear mucosa. However, previous studies have been mainly performed in COM and tumor samples, while no studies are currently available on the HPV infection in normal middle ear mucosa.

Therefore, in this study, HPV sequences, viral DNA load and HPV genotypes were investigated in middle ear specimens from patients affected by COM, including CSOM and COMC, as well as in normal middle ear specimens (NME).

2. Results

2.1. HPV DNA detection

HPV sequences were investigated by quantitative PCR (qPCR) in COM specimens (n=52), including CSOM (n=38) and COMC (n=14), as well as in NME specimens (n=56). Overall, HPV DNA was detected in 23% (12/52) of COM and 30.4% (17/56) NME specimens ($p>.05$; Figure 1). Specifically, 26.3% (10/38) of CSOM and 14.3% (2/14) of COMC tested positive for HPV sequences ($p>.05$; Figure 1).

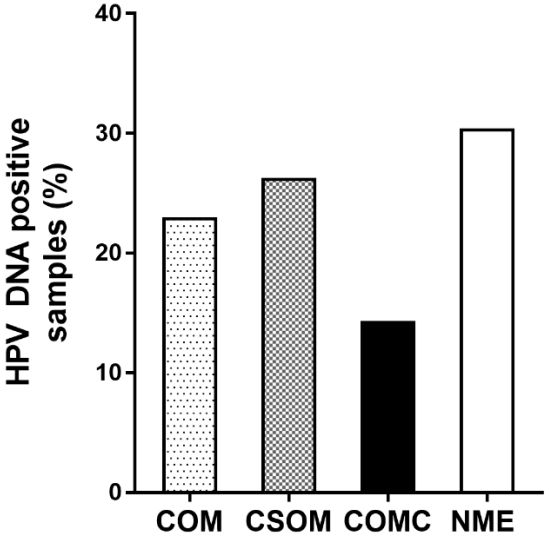

Figure 1. **Prevalence of HPV DNA in middle ear mucosa specimens**. The presence of HPV DNA was investigated in chronic otitis media specimens (n=52) (COM), including chronic suppurative otitis media (n=38) (CSOM) and chronic otitis media with cholesteatoma (n=14) (COMC) as well as normal middle ear specimens (n=56) (NME). No statistically significant differences were observed within groups (P>0.05).

2.2. HPV DNA load

HPV DNA load from middle ear mucosa specimens was determined by qPCR (Figure 2, Table 1). The mean of HPV DNA load was 2.09 copy/cell (range 0.01-8.67 copy/cell) in COM (n=12) and 1.18 copy/cell (range 0.20-1.96 copy/cell) in NME (n=17) specimens (Figure 2).

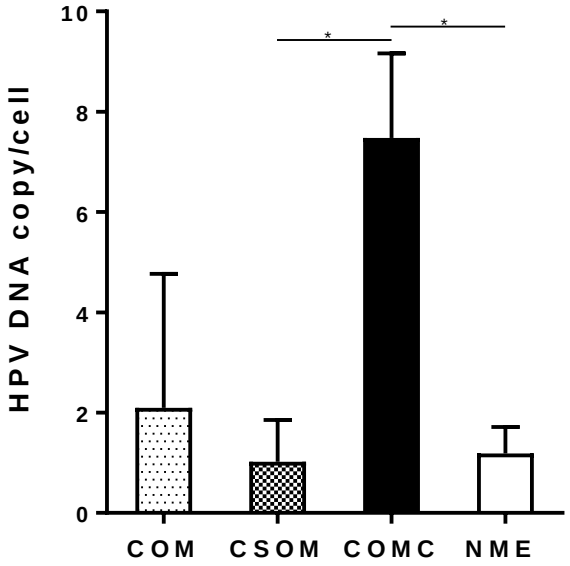

Figure 2. **Mean HPV DNA load detected by qPCR analysis.** The mean HPV DNA load (viral DNA copy/cell) was determined in HPV-positive chronic otitis media specimens (n=12) (COM), including chronic suppurative otitis media (n=10) (CSOM) and chronic otitis media with cholesteatoma (n=2) (COMC) as well as normal middle ear specimens (n=17) (NME). Error bars represent standard error of mean. *P<.05 versus CSOM and NME.

Specifically, the mean HPV DNA load was 1.02 copy/cell (range 0.01-2.36 copy/cell) in CSOM (n=10) and 7.47 copy/cell (range 6.28-8.67 copy/cell) in COMC (n=2) specimens. The difference of the HPV DNA load between COMC and CSOM as well as between COMC and NME was statistically significant (P=.03 and P=.017 versus CSOM and NME, respectively, Table 1). Although the number of COMC was very limited in sample size (n=14), it was interesting to verify that the two HPV-positive COMC samples carried a viral DNA load three- and four-fold higher than the highest viral DNA load detected in the HPV-positive CSOM and NME samples, respectively.

Table 1. Mean HPV DNA load in middle ear mucosa specimens revealed by qPCR analysis.

Middle ear mucosa specimens	Number of patients	Mean HPV DNA load (copy/cell)	Range (copy/cell)
COM	12	2.09	0.01-8.67
CSOM	10	1.02	0.01-2.36
COMC	2	7.47 † ‡	6.28-8.67
NME	17	1.18	0.20-1.96

COM: chronic otitis media; CSOM: chronic suppurative otitis media; COMC: chronic otitis media with cholesteatoma; NME: normal middle ear. † *P=.030 versus CSOM;* ‡*P=.017 versus NME.*

2.3. HPV genotyping

HPV genotypes were determined by direct sequencing analysis in HPV-positive (n=29) middle ear mucosa specimens. Twenty-nine qPCR products, from CSOM (n=10), COMC (n=2) and NME (n=17) were sequenced. DNA sequencing confirmed the presence of HPV in all specimens analyzed. HPV genotypes belonged to HPV16, HPV18, and HPV11. Specifically, HPV16 genotype was present in 100% (2/2) COMC, 50% (5/10) CSOM and 52% (9/17) NME, while HPV18 genotype was detected in 20% (2/10) CSOM and 23.5% (4/17) NME. HPV11 genotype was detected in 20% (2/10) and 5.9% (1/17) CSOM and NME, respectively. The simultaneous presence of HPV16 and HPV18 genotypes was detected in 10% (1/10) and 17.6% (3/17) CSOM and NME, respectively.

2.4. Association between HPV and co-factors

To evaluate the involvement of HPV co-factors in the etiopathogenesis of COM, a univariate analysis was performed between HPV-positive specimens and age, smoke and gender (Table 2). Results indicated that the HPV prevalence was higher in CSOM patients aged ≥65 yrs compared to age-matched NME and to CSOM patients aged ≤64 yrs (P=.006 and P=.020 versus NME and CSOM, respectively; Table 2). Moreover, HPV prevalence was higher in NME specimens from smokers than in NME from non-smokers (P=.037; Table 2).

Table 2. Association between HPV and co-factors in CSOM, COMC and NME.

PATIENTS	CSOM	COMC	NME
Age (yrs)			
≤64	5/22 (23)	1/14 (7)	16/44 (36)
≥65	10/16 (63) †‡	0/0 (0)	1/12 (8)
Smoke			
Yes	4/10 (40)	1/6 (17)	5/7 (71) §
No	6/28 (21)	0/8 (0)	12/49 (25)
Sex			
M	5/20 (25)	1/6 (17)	6/20 (30)
F	5/18 (28)	0/8 (0)	11/36 (31)

CSOM: chronic suppurative otitis media; COMC: chronic otitis media with cholesteatoma; NME: normal middle ear; †P=.020 versus CSOM aged ≤64 yrs; ‡P=.006 versus NME aged ≥65 yrs; §P=.037 versus NME no-smokers.

3. Discussion

In this study, HPV DNA sequences, viral DNA loads and genotypes were investigated in chronic otitis media (COM) specimens, including chronic suppurative otitis media (CSOM) and chronic otitis media with cholesteatoma (COMC), as well as in normal middle ear (NME) specimens.

CSOM is a middle ear disease, which depends on chronic inflammation. The aetiology of CSOM is largely unknown. High- and low-risk HPV sequences in 30.7% of CSOM have been previously reported [4]. Similarly, in the present study, 26% CSOM specimens tested positive for HPV, including high-risk and low-risk HPV genotypes, i.e. HPV16, HPV18 and HPV11. As HPV prevalence, viral DNA load and genotypes were similar in CSOM and NME specimens, no association between HPV and CSOM was found. However, a significant higher HPV prevalence was detected in CSOM patients ≥65 years old than in CSOM patients ≤65 years old, suggesting that HPV infection and old age may be co-factors in the etiopathogenesis of CSOM. One explanation is that HPV infection may persist longer in the middle ear mucosa of older than younger individuals, as shown for HPV infection in uterine cervix [30]. Considering the already known promoting role of the high-risk HPV infection in inflammation, our results suggest that the high-risk HPV infection may play a role in triggering inflammation of the middle ear mucosa in the elderly, ultimately leading to the development of CSOM. Thus, high-risk HPV infection of middle ear may explain, at least in part, in a subset of COM, the etiopathogenesis of CSOM.

COMC is a disorder of the middle ear consisting of keratinizing squamous cell epithelium. Studies on HPV in COMC are widely different in prevalence rates, ranging from 3% to 70% [19,31,32], with some reporting detection of only low-risk HPVs, HPV6 and HPV11 [3,10], and others both low-risk and high-risk HPVs, mainly HPV16 [3]. In our study, the HPV prevalence was 14% in COMC, and the high-risk HPV16 was the only genotype detected. Interestingly, the HPV16 DNA load was significantly higher in COMC (mean 7.47 copy/cell) compared to CSOM (mean 1.02 copy/cell) and NME (mean 1.18 copy/cell). This result may reflect the higher proliferation rate of HPV-infected cells from COMC compared to CSOM and NME, suggesting that viral DNA replication may occur in COMC. It has been reported that HPV infection leads to the expression of the viral oncoproteins E6 and E7, which stimulate cell growth and viral DNA replication [22,33–37]. Although no studies are available on HPV DNA load in normal and pathological middle ear tissues, our results are in agreement with previous uterine cervix investigations reporting mean of viral DNA copy/cell from 0.1 to 18 in cervical intraepithelial neoplasia and less than one viral DNA copy/cell in normal cervical tissues [37,38]. Thus, our data support and extend previous results confirming that HPV sequences can be detected in COMC. Moreover, the increased viral DNA load in COMC compared to CSOM and NME provides indirect evidence of an active infection, suggesting that HPV may play a role in the development of COMC.

The role of the HPV infection in middle ear diseases onset remains to be elucidated. In this context, it should be recalled that some HPV-infected subjects, due to their genetic or immunological characteristics, are more prone/susceptible to the viral activity leading to diseases, as reported for the HPV-positive uterine cervix tissues [30]. It is possible that older patients, due to the immune senescence, do not react properly to HPV infection in the middle ear mucosa, thus favoring with the higher HPV DNA load the inflammation process and the disease onset/progression.

Notably, it cannot be excluded that the presence of concomitant pathogenic agents, during HPV infection, could potentially increase the host susceptibility in developing inflammatory middle ear diseases. Indeed, a number of case–control studies reported an association between pathogenic infectious agents, such as *Staphylococcus aureus* [39,40] and *Pseudomonas aeruginosa* [41–43] with inflammatory middle ear diseases. Further studies based on the investigation of viral and bacterial co-infections may clarify this issue.

177 The difference in HPV DNA prevalence, among available studies, could be related to (i)
178 differences in sampling, i.e. frozen storage, as in our study vs formalin fixed [10,19–21]; (ii) storage
179 conditions, (iii) method sensitivity for HPV DNA isolation and PCR detection [10,19,20]. Further
180 studies with a larger sample size including cancer tissues are needed to assess the role of the high-
181 risk HPV infection in COMC.
182 In this study, for the first time, HPV sequences were detected in NME. Indeed, HPV sequences
183 were found in 30% of NME, indicating that the middle ear mucosa is an additional epithelial tissue
184 susceptible to the HPV infection. High-risk and low-risk HPV genotypes, i.e. HPV16, HPV18 and
185 HPV11, were detected in the middle ear mucosa. This result is in agreement with previous studies,
186 which reported HPV infection in several normal tissues [25,44]. Although middle ear epithelium is
187 not considered the primary target tissue of HPV, factors such as inflammation and smoke can trigger
188 the development of metaplastic tissue, which is the preferred target tissue of the viral infection. It is
189 well recognized that metaplasia arises in the middle ear during acute and chronic events triggered
190 by infection agents or smoke. Accordingly, in our study HPV sequences were found to be present at
191 higher prevalence in NME specimens from smokers than in NME from non-smokers, indicating that
192 smoke may favor HPV middle ear infection.

193 **4. Materials and Methods**

194 *4.1. Patients and specimens*

195 Middle ear mucosa specimens were collected from patients (n=52) suffering of COM (mean age
196 ± standard deviation [SD], 47.6±16.2 yrs), including CSOM (n=38) (mean age ± [SD], 53.7 ± 18.8 yrs)
197 and COMC (n=14) (mean age ± [SD], 41.3 ± 13.6 yrs). NME specimens (n=56) (mean age±[SD],
198 44.2±19.4 yrs) were collected from patients undergoing ear surgery for cochlear implantation or
199 stapedoplasty. Exclusion criteria were: no previous ear surgery. Middle ear mucosa specimens were
200 collected during surgery, or by middle ear swab through a tympanic perforation with a micro-
201 otoscopy. The study was performed in accordance with the Declaration of Helsinki (2008).
202 Institutional Review Board (IRB) approval was obtained from University Hospital of Ferrara Ethical
203 Committee (Authorization n. 160986, December 12th, 2016). Informed written consents were obtained
204 from patients.

205 *4.2. DNA isolation*

206 DNA was isolated according to standard procedures [45,46]. Briefly, tissue specimens were
207 incubated overnight with 100 ng/μl of proteinase K at 56°C to allow tissue digestion [47]. DNA was
208 isolated using a QIAmp DNA Blood and Tissue Extraction Kit (Qiagen, Milan, Italy) [48]. For control,
209 DNAs were extracted together with a sample of salmon sperm DNA and a mock sample lacking
210 DNA. After purification, DNA was quantified by spectrophotometric reading (NanoDrop 2000,
211 Thermo Scientific). DNA suitability for PCR analysis was evaluated amplifying the *β-globin* gene [18].
212 DNA samples were then stored at -80ºC until the time of the analysis.

213 *4.3. Viral DNA load quantification*

214 HPV DNA load was quantified by quantitative PCR (qPCR) assay, using SYBR green, with the
215 CFX96 Touch™ RT-PCR Detection System (Bio-Rad, Segrate, Milan, Italy). DNA samples were
216 analyzed by qPCR for HPV DNA sequences using the universal primer pair GP5+/GP6+, as reported
217 before [49]. Briefly, 50 ng of human genomic DNA were used in 10 μl qPCR reactions, including 2x
218 of the SsoAdvanced Universal SYBR Green Supermix, Bio-Rad (Hercules, CA, USA) and 0.5 μM of
219 each primer. PCR thermal conditions were: an initial step at 95ºC for 5 min and 40 cycles at 95ºC for
220 15s and 60ºC for 30s [49]. Each qPCR experiment was carried out using the recombinant plasmid
221 vector containing the complete HPV16 genome (NC_001526.4), used as positive control. A standard
222 curve was employed using 10-fold dilutions, from 10^8 to 10 copies, of specific recombinant plasmid
223 to calculate the viral DNA load [50]. Human *β-globin* gene was used to determine the human cell
224 equivalents of each sample under qPCR analysis. HPV DNA load values were reported as viral copies

per human cell equivalents (copy/cell). Negative controls were the two samples used during the DNA extraction, i.e. salmon sperm DNA and mock samples, and two qPCR controls, including HPV free human DNA and a non-template control [51]. Samples were run in triplicate for each qPCR assay. Experiments were run three times by different operators.

4.4. HPV genotyping

HPV genotypes were determined by direct sequencing analysis in HPV-positive middle ear samples carrying at least 1 copy/cell of viral DNA. qPCR amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) [52]. Purified qPCR amplicons were sequenced with automated ABIPrism 3730xl Genetic Analyser (Applied Biosystems) [53]. The resulting HPV DNA sequences were BLAST versus HPV DNA belonging to different viral strains present in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) [54].

4.5. Statistical analysis

The prevalence of HPV DNA in CSOM, COMC and NME specimens was evaluated by a two-sided chi-square test. Viral DNA load values were analyzed with the D'Agostino-Pearson test and means were compared with the non-parametric Kolmogorov e Smirnov test. Univariate analysis was employed to compare the features of CSOM, COMC and NME patients, such as age, gender and smoke, in association with HPV. Statistical analyses were performed using Graph Pad Prism version 5.0 for Windows (Graph Pad, La Jolla, CA, USA) [55,56]. P-values <.05 were considered statistically significant [57].

5. Conclusions

In conclusion, this study shows that HPV sequences are present in CSOM, COMC and normal middle ear specimens. We also show that the high risk HPV16 and HPV18 are the main genotypes detected in CSOM, COMC and NME specimens. Lastly, although HPVs have been detected in CSOM, COMC and NME with similar prevalence, high risk HPV DNA load was higher in COMC compared to CSOM and NME. Altogether these data indicate that CSOM and COMC epithelia and normal middle ear mucosa are target tissues of HPV infection. It remains to be assessed whether the higher HPV DNA load detected in COMC is significant for a putative pathogenic role of HPV in this middle ear disease.

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